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Development of a novel biomaterial: Part II. Evaluation of a photo cross-linking method

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Defence R&D Canada – Toronto
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Abstract

This report describes the process for photo cross-linking the components of a biopolymer-elastomer interpenetrating polymer network (IPN) biomaterial for use as a wound dressing. Cross-linking of methacrylated gelatin was performed by ultraviolet irradiation in the presence of a photoinitiator. The yield and extent of the gelatin methacrylation reaction were quantified using various methods. Unexpectedly, we determined that HydroThane™ also cross-linked during the polymerization process, suggesting that a full IPN may be formed during the ultraviolet irradiation of the pre-IPN gelatin-HydroThane™ mixtures. Photo cross-linking of pre-IPN mixtures containing methacrylated gelatin and HydroThane™ produced films with desirable swelling ratios for our intended application. Several factors were examined for their contribution in determining the absorbency and/or mechanical strength of the films. These factors include: concentration of the photoinitiator; concentrations and component fractions of the different polymers; and, mixing conditions of the pre-IPN mixture. We also determined that antiseptic-loaded photo cross-linked IPN films remained bactericidal for up to 3 days. Lastly, the production of IPN films was scaled-up to enable future *in vitro* and *in vivo* testing of IPN films. In summary, further development of the gelatin-HydroThane™ IPN biomaterial as a medicated wound dressing for treating deep, hemorrhagic cavity injuries sustained on the battlefield appears promising.

Résumé

Dans cet article, on décrit un procédé de photo-réticulation des composants d'un biomatériau constitué d'un réseau polymère interpénétrant (RPI) de biopolymère-élastomère, destiné à être utilisé comme pansement. La gélatine méthacrylatée a été réticulée par exposition à un rayonnement ultraviolet en présence d'un photoamorceur. Le rendement et l'ampleur de la réaction de méthacrylation de la gélatine ont été quantifiés à l'aide de diverses méthodes. Fait inattendu, nous avons constaté qu'il y avait aussi réticulation de l'HydroThane^{MD} durant la polymérisation, ce qui permet de penser qu'il y a peut-être formation d'un RPI complet durant l'exposition au rayonnement ultraviolet des mélanges gélatine-HydroThane^{MD} pré-RPI. La photo-réticulation de mélanges pré-RPI contenant de la gélatine méthacrylatée et de l'HydroThane^{MD} a donné des pellicules présentant des rapports de gonflement souhaitables pour l'application prévue. On a examiné plusieurs facteurs afin de déterminer dans quelle mesure ils influaient sur le pouvoir absorbant et/ou la résistance mécanique des pellicules. Parmi ces facteurs, on compte la concentration du photoamorceur, les concentrations des différents polymères et les fractions de leurs constituants, et les conditions dans lesquelles est traité le mélange pré-RPI. Nous avons également constaté que les pellicules RPI photo-réticulées chargées d'un antiseptique conservaient leur activité bactéricide pendant une période pouvant atteindre trois jours. Enfin, les pellicules RPI ont été fabriquées à une plus grande échelle, afin de permettre la réalisation d'essais *in vitro* et *in vivo* ultérieurs. En résumé, la poursuite du développement du biomatériau à RPI constitué de gélatine-HydroThane^{MD}, en vue de produire un pansement médicamenteux destiné au traitement des blessures profondes subies sur le champ de bataille, comportant une cavité et risquant d'être accompagnées d'une hémorragie, donnera, semble-t-il, des résultats prometteurs.

Executive summary

Wound injuries are a predictable and potentially life-threatening outcome of front-line military operations. Most superficial wounds are relatively straightforward to treat using conventional dressings. However, one important operational challenge is to treat heavily hemorrhagic cavity wounds, especially those in hard-to-compress areas such as the groin and shoulder. Under a Technology Investment Fund, DRDC Toronto has undertaken the development of a novel gelatin-HydroThane™ material for use as a wound dressing for treating cavity and secondary blast injuries sustained on the battlefield. This report describes the process for photo cross-linking the components of a biopolymer-elastomer interpenetrating polymer network (IPN) biomaterial.

One of the critical steps in the preparation of the composite biomaterial is that gelatin and HydroThane™ have such different properties that they tend to phase-separate upon their mixing in a common solvent. Our proposed methodology for circumventing phase-separation is by intimately mixing gelatin and HydroThane™ during the material preparation, and rapidly introducing cross-linking into the system to maintain and stabilize this level of mixing before phase-separation or polymer domain formation occurs. Thus, cross-linking of methacrylated gelatin was performed by ultraviolet irradiation in the presence of a photoinitiator. The yield and extent of the gelatin methacrylation reaction were quantified using various methods, such as iodobromine titration and Fourier Transformed Infrared spectroscopy. Unexpectedly, we determined that HydroThane™ also cross-linked during the polymerization process. This finding suggests that a full IPN may be formed during the ultraviolet irradiation of the pre-IPN gelatin-HydroThane™ mixtures, with both polymers being cross-linked.

Photo cross-linking of pre-IPN mixtures containing methacrylated gelatin and HydroThane™ produced films with desirable swelling ratios, typically above those of commercially available wound dressings. In an attempt to optimize the formulation of the biomaterial, several factors were examined for their contribution in determining the hydration and/or mechanical strength of the films. These factors include: component fractions of the different polymers; method for mixing the pre-IPN mixture; and, concentration of the photoinitiator. Using a standard *in vitro* microbiological assay, we also determined that the bactericidal activity of antiseptic-loaded photo cross-linked IPN films was superior to that of a commercial polyurethane-based wound dressing. Lastly, the production of IPN films was scaled-up to enable future *in vitro* and *in vivo* testing.

In summary, synthesis of biopolymer-elastomer composites via a photo cross-linking process was successfully achieved. Further development of the gelatin-HydroThane™ IPN biomaterial as a medicated wound dressing for treating deep, hemorrhagic cavity injuries sustained on the battlefield appears promising.

Martineau, L., Peng, H.T., and Shek, P.N. 2005. Development of a novel biomaterial. Part II: Evaluation of a photo cross-linking method. DRDC Toronto TR 2005-201. Defence R&D Canada – Toronto.

Sommaire

Les blessures sont des résultats prévisibles et potentiellement mortels d'opérations militaires menées en première ligne. On peut traiter relativement facilement les blessures superficielles à l'aide de pansements classiques. Toutefois, le traitement de blessures comportant une cavité qui sont accompagnées d'une grave hémorragie, plus particulièrement celles situées dans des endroits où l'on peut difficilement appliquer une pression, comme l'aine et l'épaule, constitue un important défi à relever dans un contexte opérationnel. Grâce au financement obtenu dans le cadre du Fonds d'investissement technologique, RDDC Toronto a entrepris la mise au point d'un nouveau matériau de type gélatine-HydroThane^{MD} destiné à être utilisé comme pansement sur les blessures comportant une cavité et sur les lésions secondaires par souffle subies sur le champ de bataille. Dans cet article, on décrit un procédé de photo-réticulation des composants d'un biomatériau constitué d'un réseau polymère interpénétrant (RPI) de biopolymère-élastomère.

Le préparation du biomatériau composite comporte une étape cruciale au cours de laquelle la gélatine et l'HydroThane^{MD}, en raison de leurs propriétés si différentes, ont tendance à se séparer en deux phases lorsqu'ils sont mélangés dans un même solvant. Pour prévenir la séparation en phases, nous proposons de mélanger intimement la gélatine et l'HydroThane^{MD} durant la préparation, puis de procéder rapidement à la réticulation du système pour maintenir et stabiliser ce mélange avant qu'il n'y ait séparation en phases ou formation du polymère. La gélatine méthacrylatée a donc été réticulée par exposition à un rayonnement ultraviolet en présence d'un photoamorceur. Le rendement et l'ampleur de la réaction de méthacrylation ont été quantifiés à l'aide de diverses méthodes, telles que le titrage par l'iodure de brome et la spectroscopie infrarouge à transformée de Fourier. Fait inattendu, nous avons constaté qu'il y avait aussi réticulation de l'HydroThane^{MD} durant la polymérisation, ce qui permet de penser qu'il y a peut-être formation d'un RPI complet durant l'exposition au rayonnement ultraviolet des mélanges gélatine-HydroThane^{MD} pré-RPI et, ainsi, réticulation des deux polymères.

La photo-réticulation de mélanges pré-RPI contenant de la gélatine méthacrylatée et de l'HydroThane^{MD} a donné des pellicules présentant des rapports de gonflement souhaitables, qui étaient typiquement supérieurs à ceux des pansements disponibles dans le commerce. Afin d'optimiser la préparation du biomatériau, nous avons examiné plusieurs facteurs pour déterminer dans quelle mesure ils influaient sur l'hydratation et/ou la résistance mécanique des pellicules. Parmi ces facteurs, on compte les fractions des constituants des différents polymères, la méthode de mélange du mélange pré-RPI, et la concentration du photoamorceur. Grâce à une épreuve microbiologique *in vitro* couramment utilisée, nous avons également déterminé que les

Martineau, L., Peng, H.T. et Shek, P.N. 2005. Mise au point d'un nouveau biomatériau. Partie II : Évaluation d'une méthode de photo-réticulation. RDDC Toronto TR 2005-201. R & D pour la défense Canada – Toronto.

pellicules RPI photo-réticulées chargées d'un antiseptique possédaient une activité bactérienne supérieure à celle des pansements à base de polyuréthane disponibles dans le commerce. Enfin, les pellicules RPI ont été fabriquées à une plus grande échelle, afin de permettre la réalisation d'essais *in vitro* et *in vivo* ultérieurs.

En résumé, nous avons procédé avec succès à la synthèse par photo-réticulation de composites constitués d'un biopolymère et d'un élastomère. La poursuite du développement du biomatériau à RPI constitué de gélatine-HydroThane^{MD}, en vue de produire un pansement médicamenteux destiné au traitement des blessures profondes subies sur le champ de bataille, comportant une cavité et accompagnées d'une hémorragie, donnera, semble-t-il, des résultats prometteurs.

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1. Introduction

Wound injuries are a predictable and potentially life-threatening outcome of front-line military operations. Most superficial wounds (including burn injuries) are relatively straightforward to treat using conventional dressings. However, one important operational challenge is to treat heavily hemorrhagic cavity wounds, especially those in hard-to-compress areas such as the groin and shoulder. Besides the severity of the wounds sustained, and considering the fact that all wounds are deemed painful and contaminated, management of combat wounds offers in itself challenges that are different from treating comparable civilian wounds. For example, wound care may need to be self-administered or by untrained personnel in extreme conditions. A combat wound dressing must therefore be simple; compact yet markedly expandable, thus allowing the packing of different wound sizes and depths; lightweight; easy-to-use; and, capable of providing sustained delivery of therapeutic agents (e.g., hemostatic agents; antimicrobials; and/or pain killers).

Under research contract W7711-027745/001, Dr. Josephine Turner (Dorset Technologies Ltd., Kettleby, ON) performed extensive searches of the patent databases as well as scientific literature in the field of wound care materials. These led to the concept of a novel biopolymer-elastomer material with key attributes for addressing the requirements for treating cavity and secondary blast injuries sustained on the battlefield (1). A first attempt to produce an experimental prototype of the biopolymer-elastomer material was recently reported (2). Briefly, of all the biopolymers and elastomers tested, only gelatin and HydroThane™ could be dissolved in a common organic solvent, that is, dimethylsulfoxide. A series of experiments were then carried out to identify the chemical cross-linking system compatible with both gelatin and HydroThane™ in dimethylsulfoxide. Use of 1-ethyl-3(3-dimethyl aminopropyl) carbodiimide to cross-link gelatin failed to produce any gelatin-HydroThane™ films. A method for producing glutaraldehyde (GTA) cross-linked gelatin-HydroThane™ films was thereafter successfully developed. However, the ability of the GTA cross-linked gelatin-HydroThane™ films to absorb serum, and by extension any fluid such as wound exudates, was only about half of our target requirement for use of the biopolymer-elastomer material as a combat wound dressing. This data, taken together with the facts that: the cross-linked network was not homogeneous; the GTA cross-linking reaction was not stable; and, degradation of the GTA cross-link may result in release of potentially cytotoxic by-products, led us to abandon this experimental approach for cross-linking gelatin for the preparation of the biopolymer-elastomer material, and to recommend using alternate methods for effectively cross-linking the selected polymers.

The present technical report describes the development of a photo-cross-linking method for preparing the gelatin-HydroThane™ material. Most of the experimental results were obtained in the laboratories of DRDC Toronto under contract W7711-027800/001 (Dorset Technologies, Kettleby, ON). R&D activities were performed in support of MOM Thrust 6c under WBE 16ci04 (previously 16ca37; Emerging Material Technologies for Applications in Battlefield Wound Care).

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2. Methacrylation of gelatin

One of the critical steps in the preparation of our biopolymer-elastomer material is related to the fact that the two components (i.e., gelatin and HydroThane™) have such different properties that they tend to phase-separate upon mixing in a common solvent (i.e., dimethylsufoxide, DMSO) or upon hydration of the material. Our proposed methodology for circumventing phase-separation is by intimately mixing gelatin and HydroThane™ in a solution during the material preparation, and rapidly introducing cross-linking into the system to maintain and stabilize this level of mixing before phase-separation or polymer domain formation occurs. The resultant interpenetrating polymer network (IPN) material formed will therefore contain very small domain sizes of each component polymer. The small domain sizes are a desirable characteristic; they are an attribute of a well-mixed polymer blend in which each polymer component maintains its respective properties, but also acts synergistically with the other polymer to enhance the material's properties.

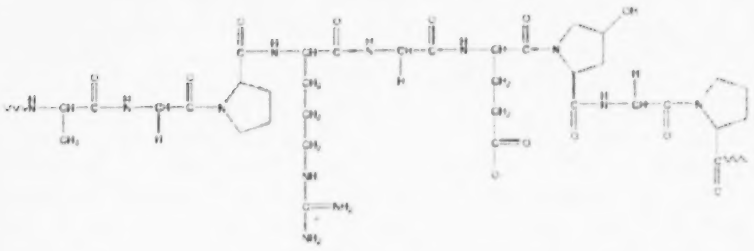
Considering our requirement that the cross-linking of gelatin be precisely controlled in terms of when the cross-linking reaction is initiated, it appeared that the most effective strategy for initiating the cross-linking of gelatin films was to use an external energy source such as ultraviolet (UV) irradiation. The rationale was that such a system would allow thorough mixing of all compounds in the gelatin-HydroThane™-DMSO reaction mixture well before the cross-linking reaction is initiated. A literature review was therefore carried out to determine which methods are currently available for photo cross-linking gelatin (3-6). Typically, gelatin is methacrylated before the UV cross-linking procedure is initiated (7, 8).

2.1 Experimental procedures

There are two types of gelatin. More specifically, Type A is derived from acid-processed materials, primarily pork skin, while Type B is derived from alkaline- or lime-processed materials, primarily cattle or calf hides and ossein (i.e., bone collagen). Methacrylation of either type of gelatin introduces vinyl groups into the gelatin molecule by esterification of the amino and hydroxyl groups in gelatin (Fig. 1A) with either glycidyl methacrylate (Fig. 1B) or methacrylic anhydride (Fig. 1C). Following this trans-esterification reaction, the vinyl groups can polymerize via UV irradiation in the presence of a photoinitiator. Briefly, photoinitiators incorporated into the reacting mixture (e.g., 2, 2-dimethoxy-2-phenylacetophenone) split upon exposure to UV light, and the pair of electrons released from the broken bond forms simultaneously two types of initiator fragments or free radicals (Fig. 2). The benzoyl radicals then break the double bonds in the vinyl groups to produce free radicals that may then attack other vinyl groups to transfer the radicals from one site to another. Termination of the reaction occurs when free radical strips a hydrogen atom from an active chain, a process called disproportionation. Alternately, termination may occur via recombination when two free radicals in the reacting mixture join together, a process resulting in the cross-linking of gelatin. This cross-linking procedure involves strict

control of the reaction temperature as well as UV intensity. Figure 3 depicts the photo cross-linking process leading to the formation of the gelatin-HydroThane™ IPN biomaterial.

A.



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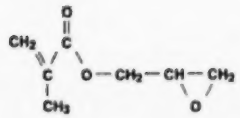

C.

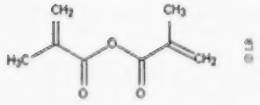

Figure 1. *Chemical structures of gelatin (Panel A), glycidyl methacrylate (Panel B), methacrylate anhydride (Panel C).*

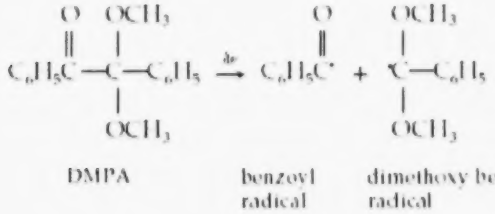

Figure 2. *Photodecomposition of the photoinitiator 2, 2-dimethoxy-2-phenylacetophenone (DMPA) and its free radicals.*

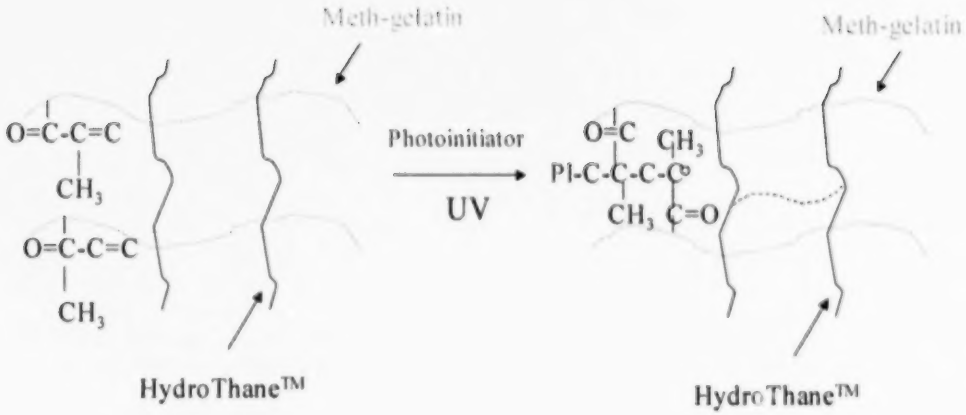

Figure 3. *Schematic of the photo cross-linking process leading to the formation of the gelatin-HydroThaneTM IPN biomaterial.*

2.1.1 Methacrylation of gelatin using methacrylic anhydride

The methacrylation of gelatin was carried out using methacrylic anhydride (Aldrich, Mississauga, ON), using modified procedures (8, 9). Briefly, 10 g of gelatin (Type A, Bloom strength of 235; Great Lakes Gelatin Inc., Grayslake, IL) were dissolved in 100 mL of either phosphate buffered saline (PBS) or DMSO, and stirred at 50°C. After complete solubilization of the gelatin, 12 mg of dimethylaminopyridine (DMAP; a catalyst) and 0.6 mL of triethylamine (TEA; an acid trap) were added to some of the gelatin mixtures to assess their usefulness for the methacrylation procedures. All gelatin mixtures were then supplemented with either 0.5 or 1.0 mL methacrylic anhydride. These samples are referred to as 1 MAAH and 2 MAAH, respectively. As methacrylic anhydride is highly moisture-sensitive, care was taken to handle the chemical under nitrogen in a glove box to prevent its hydrolysis to methacrylic acid, which would have led to reduced reactivity with the amino and hydroxyl groups on gelatin. The mixture of gelatin and methacrylic anhydride was stirred for 60 min at 50°C. After cooling the mixture at room temperature, the methacrylated gelatin was precipitated out of DMSO overnight using 100 mL of ethanol. The precipitate was then dialyzed (Fisherbrand dialysis membranes, MWCO 12,000-14,000 D) against double distilled water for up to 7 days either at room temperature or at 35-40°C to remove unreacted methacrylic anhydride from the solution. Water changes were performed twice daily during the 7-d washing period, and the UV absorbance of the wash water was recorded at various wavelengths

(UV/Vis Spectrophotometer, Model G1103A, Agilent Technologies Deutschland GmbH, Waldbronn, Germany) to assess the release of DMSO from the methacrylated gelatin solution during the dialysis period. Once all non-reacted components were removed, the methacrylated gelatin solution was lyophilized for 5 days, after which period the dry weight of the methacrylated gelatin was recorded. The reaction yield of the methacrylation procedure was calculated as the difference between the final dry weight of methacrylated gelatin and the initial dry weight of gelatin.

2.1.2 Methacrylation of gelatin using glycidyl methacrylate

Methacrylation of gelatin was also carried out using glycidyl methacrylate (Aldrich, Mississauga, ON), using a modification of the procedure described by Koepff et al. (7). Briefly, 10 g of gelatin were dissolved in 90 mL of PBS, and stirred at 50°C. The pH of the mixture was then adjusted to 8.5 using 1 N NaOH. After complete solubilization of the gelatin, 0.45 mL of glycidyl methacrylate was added, and the mixture was stirred for 60 min at 50°C. The pH was then adjusted to 7 using 2 N H_2SO_4. The methacrylated gelatin was then precipitated out of DMSO using approximately 100 mL of ethanol, and dialyzed against double distilled water as previously described. Water changes were performed twice daily during the 6-d period. Once all non-reacted components were removed, the methacrylated gelatin solution was lyophilized for 5 days, after which period the dry weight of the methacrylated gelatin was recorded. The reaction yield of the methacrylation procedure was calculated as described in Section 2.1.1.

2.2 Yield of the gelatin methacrylation reaction

Table 1 shows the reaction yield of various methacrylation procedures when using methacrylic anhydride. Raising the temperature of the dialysis procedure to 35-40°C reduced the reaction yield by half, with only 33% of the initial gelatin being methacrylated. However, the yield of the reaction was restored either by doubling the initial amount of methacrylic anhydride in the reacting system or by adding a mixture containing DMAP and TEA. It is noteworthy that a comparable reaction yield of 70% was obtained when using glycidyl methacrylate (data not shown). However, preliminary attempts to prepare photo cross-linked gelatin films were not as successful when using gelatin methacrylated with glycidyl methacrylate compared to methacrylic anhydride, suggesting an incomplete methacrylation of gelatin when glycidyl methacrylate was used. The photo cross-linking process of gelatin was therefore further optimized using methacrylic anhydride.

The solubility of methacrylic anhydride in DMSO being greater than that in PBS, we expected a higher degree of methacrylation in the former solvent. Our finding of a reduction of the reaction yield when gelatin was methacrylated in DMSO (Table 1) might be related to a greater susceptibility of the DMSO-methacrylated gelatin to be removed from solution during the precipitation step than the PBS-methacrylated gelatin. Alternately, the volume of ethanol that we used to precipitate out the DMSO-methacrylated gelatin may have been insufficient, as the typical volume required is 10-fold that of the initial volume of solvent (Dr. Appleman, personal communication).

Table 1. *Reaction yield of different gelatin methacrylation procedures performed in PBS or DMSO, using methacrylic anhydride*

VOLUME METHACRYLIC ANHYDRIDE (μL/ g gelatin)	SOLVENT	DIALYSIS CONDITIONS	DMAP AND TEA	REACTION YIELD (%)
50	PBS	Room temperature for 6 days	No	72±4*
50	PBS	Heated at 35-40°C for 6 days	No	33
50	PBS	Heated at 35-40°C for 4 days	Yes	66
100	PBS	Heated at 35-40°C for 4 days	No	73
100	DMSO	Heated at 35-40°C for 6 days	No	40
500	DMSO	Heated at 35-40°C for 6 days	No	18

PBS Phosphate Buffered Saline DMAP Dimethylaminopyridine DMSO Dimethylsulfoxide TEA Triethylamine * Means ± SEM (n=5)

Figure 4 shows that DMSO was removed from solution within 5 days of dialysing at room temperature the methacrylated gelatin sample prepared in the absence of DMAP and TEA. Similar findings were observed when dialysing a methacrylated gelatin solution containing 10 times the amount of methacrylic anhydride (Fig. 5). However, it is noteworthy that increasing the amount of methacrylic anhydride seemed to postpone the release of the non-reacted components. Due to technical considerations, we did not assess the release of TEA or DMAP from any of methacrylated gelatin solutions during the dialysis period.

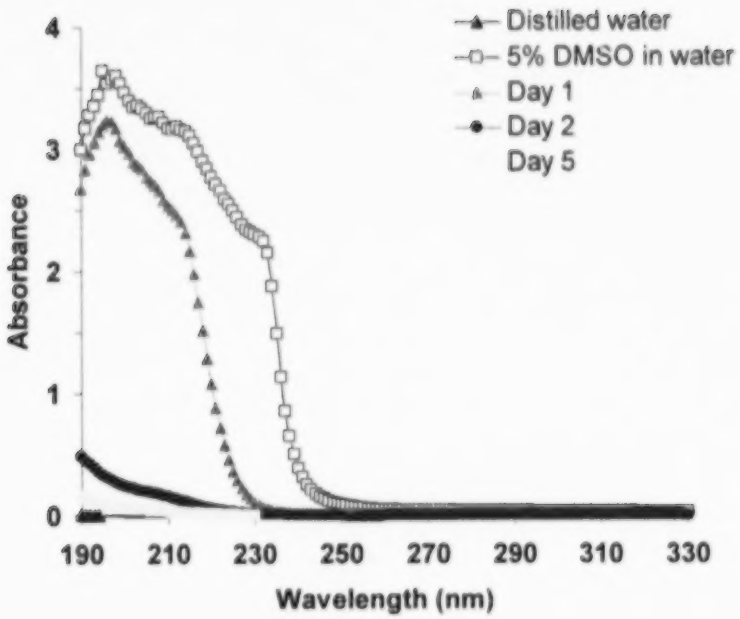

Figure 4. *UV spectrum of the wash water collected at different time intervals during the dialysis of the methacrylated gelatin solution. Gelatin was methacrylated in DMSO in the absence of DMAP and TEA using methacrylic anhydride, and dialysed at room temperature. Data represent one sample per experimental day or condition.*

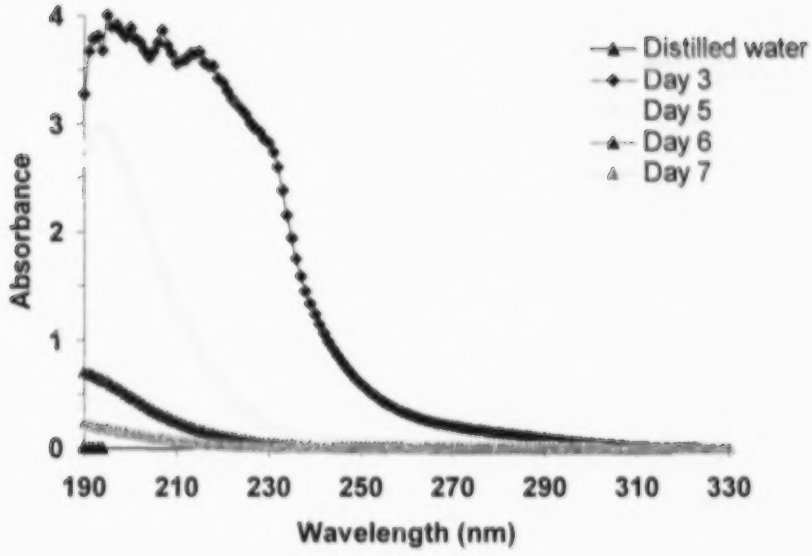

Figure 5. *UV spectrum of the wash water collected at different time intervals during the 7-d dialysis of a gelatin solution methacrylated in DMSO in the absence of DMA and TEA using 10 times the normal amount of methacrylic anhydride. Gelatin was dialysed at 35-40°C. Data represent one sample per experimental day or condition.*

2.3 Quantification of extent of gelatin methacrylation

To allow selection of the optimal conditions for the methacrylation of gelatin, one needs to know not only the yield of the reaction, but also to what extent gelatin was methacrylated. Based on a literature review, different methods were used to assess the degree of gelatin methacrylation, including UV spectrophotometry (10), an iodine titration method (11-12), and Fourier Transform Infrared Spectroscopy (13).

2.3.1 UV spectrophotometry

Aliquots of gelatin, methacrylated gelatin or methacrylic anhydride were dissolved in DMSO to a final concentration of 1% w/w, the latter concentration being chosen to ensure that the absorbance would remain below 1. The UV spectrum of the different solutions was then recorded at various wavelengths (UV/Vis Spectrophotometer, Model G1103A, Agilent Technologies Deutschland GmbH, Waldbronn, Germany). Figure 6 shows that methacrylated gelatin absorbs at the same wavelength as gelatin, therefore precluding the use of this method for the quantification of the extent of gelatin methacrylation.

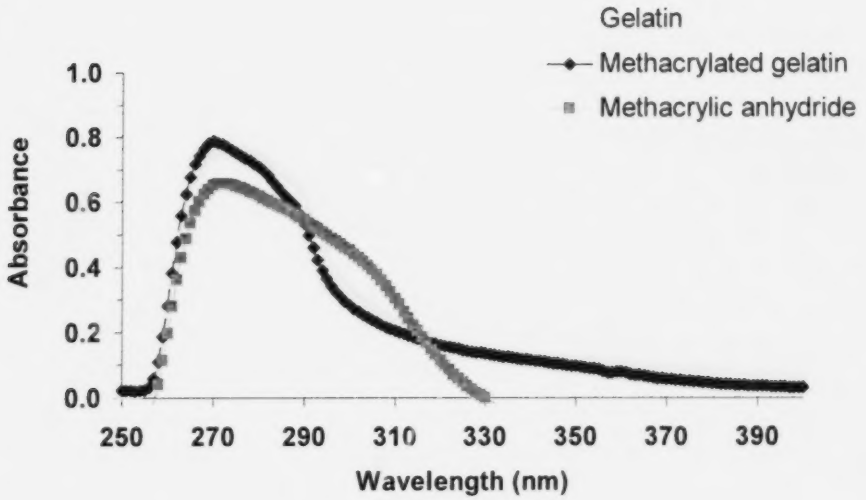

Figure 6. *UV spectra of 1% w/w solutions of gelatin, methacrylated gelatin, and methacrylic anhydride prepared in DMSO.*

It is noteworthy that methacrylated gelatin dissolved freely in DMSO at concentrations up to 18% (w/w), the consistency of the resulting solution being comparable to that of honey. Considering that dissolution of gelatin in DMSO at the same concentration yielded a solution of the consistency of an amorphous gel (2), the present data indicate that methacrylated gelatin is likely more soluble than native gelatin in DMSO, likely due to the disparition of the hydrogen bonds in the molecule following the methacrylation procedures.

2.3.2 Iodobromine titration

Published titration procedures (11, 12) were modified and scaled-down to reflect the smaller sample size as well as lower expected number of double carbon bonds in our methacrylated gelatin samples. In these procedures, iodobromine is attached to the double carbon bond of the methacrylated gelatin through addition of Hanus solution (iodobromide in acetic acid) in excess. Once the reaction is complete, excess iodobromine is reacted with iodide forming I_2, which in turn is determined by standard thiosulphate titration.

Gelatin was methacrylated in the absence of DMAP and TEA, as described in Section 2.1.1. Briefly, 1 g of gelatin was dissolved in 10 mL of DMSO or PBS, and stirred at 50°C. After complete solubilization of the gelatin, different volumes of methacrylic anhydride were added (i.e., 50, 100 or 250 µL). The mixture of gelatin and methacrylic anhydride was then: stirred for

60 min at 50°C; cooled at room temperature; precipitated out in ethanol; stirred overnight; dialyzed for 7 days at 35-40°C; and, lyophilized for 5 days.

Samples (0.05 g) of either methacrylated gelatin or pure gelatin were dissolved in DMSO to a final concentration of 7.5% w/w. The openings of the Erlenmeyer flask were then plugged with a rubber septum. After adding 50.0 µL of 0.2 M iodobromine in acetic acid, the flasks were covered with aluminum foil and placed in the dark in the fume hood for at least 60 min with occasional shaking. A 20-µL aliquot of 15% potassium iodide in distilled water was then added to each flask, and the solutions were shaken thoroughly. The reaction was completed by adding 10 mL of distilled water and 3 drops of starch indicator. Each solution was then titrated with 0.002 M sodium thiosulphate until the solution became clear; the volume of sodium thiosulphate used was then recorded. The degree of methacrylation of gelatin, defined as the ratio between the amount of lysine and hydroxyproline in methacrylated gelatin and the initial amount of lysine and hydroxyproline in pure gelatin was calculated, assuming a degree of substitution of 20% (14).

The extent of the reaction between iodobromide and gelatin was 18 times greater when the gelatin was methacrylated in DMSO than in PBS, suggesting a larger degree of methacrylation (Table 2). However, the degree of gelatin methacrylation in DMSO consistently remained below 1.6%, and was inversely related to the volume of methacrylic anhydride used for the methacrylation reaction (Table 2). This finding may be explained by our observation that the solubility of gelatin in DMSO was reduced when more than 100 µL of methacrylic anhydride was used for the methacrylation procedures. It is therefore likely that some methacrylated gelatin remained a precipitate and did not react in the titration reaction, thereby accounting for the lower substitution results. Considering that the observed methacrylation degree was approximately four times lower than that reported in the literature (1), we carried out an experiment to determine the effectiveness of the titration method.

Briefly, the titration procedure was carried out as described above, with the exception that the 0.05 g of methacrylated gelatin sample was replaced by 0.98 g of distilled water, 0.5 mL DMSO, or 1 µL of 94% methacrylic anhydride mixed in 0.5 mL DMSO. We observed that while DMSO was relatively inert, less than a third of the double bonds of methacrylic anhydride were combining with iodobromine (Table 3). This data suggested that the degree of gelatin methacrylation in DMSO calculated in Table 2 might have represented only one third of the total methacryl groups present, thus bringing our observed value to within 90% of that reported in the literature (1). However, our observation of lower degrees of methacrylation than those observed for the blanks for several of our methacrylated gelatin samples, taken together with the successful formation of gelatin films (see next section) questioned the accuracy of this titration method. We therefore investigated whether Fourier Transform Infrared Spectroscopy (FTIR) could be used as a quality control step.

Table 2. *Determination of the extent of gelatin methacrylation using a titration method*

TYPE OF GELATIN	SOLVENT	VOLUME METHACRYLIC ANHYDRIDE (μL/ g gelatin)	DEGREE OF SUBSTITUTION (%)
Native	DMSO	0	0
Methacrylated	PBS	50	0.038
Methacrylated	DMSO	50	1.61
Methacrylated	DMSO	100	1.50
Methacrylated	DMSO	250	1.21

PBS Phosphate Buffered Saline DMSO Dimethylsulfoxide

Table 3. *Effectiveness of the titration procedure for assessing the degree of gelatin methacrylation*

SOLUTE	SOLVENT	IODOBROMINE CONSUMED (%)	DOUBLE CARBON BONDS REACTED (%)
Meth-gelatin	Distilled water	9.36	N/A
Meth-gelatin	DMSO	0	N/A
MAAH	DMSO	N/A	30.43

Meth-gel Methacrylated gelatin MAAH Methacrylic anhydride DMSO Dimethylsulfoxide

2.3.3 Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared (FTIR) spectroscopy is often used to determine the structural changes in the molecules used for the preparation of biomaterials (13). 10 g of gelatin were methacrylated in 100 mL of PBS in the absence of TEA and DMAP, using either 0.5 or 1 mL of methacrylic anhydride; these samples are referred to as 1 MAAH and 2 MAAH, respectively. A sample of native gelatin was similarly dissolved in PBS, and used as a control. The infrared spectra of native and methacrylated gelatin

were recorded using a FTIR spectroscope (Thermo Electron Corporation, Model Nicolet IR 100, Mississauga, ON), as per the manufacturer's procedures.

The infrared spectrum of pure gelatin exhibited the characteristic amide absorption bands at 1640 cm^{-1} and 1530 cm^{-1} (Fig. 7). While methacrylation of gelatin using 2 MAAH caused a small shift of the amide bands of gelatin to higher frequencies, no difference in the IR spectrum was observed when using 1MAAH. These data suggest a much smaller gelatin methacrylation at lower methacrylic anhydride concentrations. Furthermore, two small bands appeared at 1720 cm^{-1} and 723 cm^{-1}, perhaps due to the introduction of ester and vinyl groups on the gelatin molecule during the methacrylation procedure (Fig. 7). Similar infrared spectra were obtained using different batches of methacrylated gelatin (data not shown), suggesting the usefulness of FTIR for confirming the effectiveness of the gelatin methacrylation procedures when preparing various batches (i.e., as a quality control step).

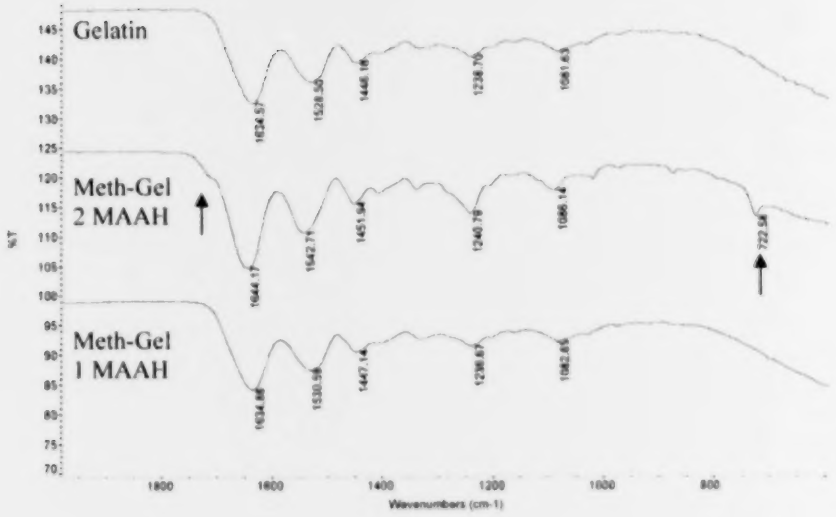

Figure 7. *Typical infrared spectra of native and methacrylated gelatin (Meth-Gel) recorded using FTIR. Gelatin was methacrylated in PBS using either 0.5 mL (1 MAAH) or 1.0 mL of methacrylic anhydride (2 MAAH). Arrows indicate the position of new bands following methacrylation of the gelatin.*

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3. Formation of photo cross-linked gelatin and HydroThane™ films

3.1 Gelatin films

A preliminary experiment was carried out to assess the amount of photo-initiator and methacrylated gelatin required to prepare photo cross-linked gelatin films. Briefly, solutions containing 5, 10 and 15% (w/w) of methacrylated gelatin were prepared in DMSO. Different aliquots of the photo-initiator 2, 2-dimethoxy-2-phenylacetophenone (Irgacure 651; Ciba Specialty Chemicals, Toronto, ON) were then added to the methacrylated gelatin solutions in a scintillation vial to reach final concentrations of 1%, 4%, 7.5% and 10% relative to the methacrylated gelatin mass. The reaction mixtures were prepared with stock solutions of 5%, 10% or 15 % (w/w) methacrylated gelatin. The mixtures were vigorously vortexed for 30 s and ultraviolet (UV)-irradiated at an intensity of 2 mW/cm^2 (Rayonet model RPR-200; Southern New England Company, Branford, CN) for 15 min at 350 nm (manufacturer's specifications). The resulting gelatin films (n=1 per experimental condition) were cooled at room temperature for 60 min, before being washed in double distilled water. Due to technical difficulties, the weight fraction of water in the swollen photo cross-linked gelatin film was only measured 30 days later, and was calculated as the ratio of the difference between wet mass and dry mass over the measured wet mass (i.e., $H_{30\text{-d wash}}$). Similarly, the swelling ratio of the photo cross-linked gelatin films ($SR_{30\text{-d wash}}$), defined as the number of times the dry film absorbs its own weight in water, was calculated as the ratio of the wet weight after 30 days over the initial dry weight.

All gelatin solutions were visually cross-linked after being UV-irradiated. However, films containing 5% methacrylated gelatin quickly dissolved upon washing, suggesting that the extent of cross-linking was minimal due to the large dilution factor of the gelatin solution, and would not be adequate for preparation of the gelatin-HydroThane™ IPN material. The hydration levels of IPN composite biomaterials are typically above 0.8 (15). $H_{30\text{-d wash}}$ of the photo cross-linked gelatin films prepared with the 10% and 15 % (w/w) methacrylated gelatin solutions ranged between 0.92 and 0.99, irrespective of the initial concentration of photo-initiator (data not shown).

Figure 8 shows the swelling ratios of gelatin films photo cross-linked with different proportions of Irgacure 651 in the reaction mixture. The $SR_{30\text{-d wash}}$ of all but one of our photo cross-linked gelatin films were 4 to 12 times greater than those measured when the gelatin films prepared using a glutaraldehyde cross-linking method (2). The latter observation further supports the use of photo cross-linking as the method of choice for forming our biopolymer-elastomer IPN material. It is noteworthy that the high swelling ratios obtained confirm the tremendous absorption capability of gelatin. However, it is unclear from the current results to what extent the presence of HydroThane™ in the pre-IPN reaction mixture or further processing the photo cross-link IPN films after washing (e.g., freeze-drying) would alter these swelling ratios.

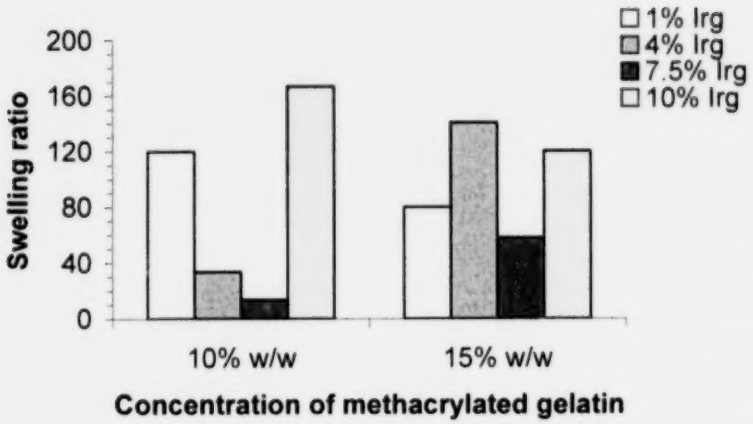

Figure 8. *Swelling ratios of photo cross-linked gelatin films prepared with different proportions of Irgacure 651 (Irg) relative to the methacrylated gelatin solution. Values were measured following a 30-d washing period in distilled water. Data represent one film per experimental condition.*

While high swelling ratios were measured for the photo cross-linked gelatin films prepared with a stock solution containing 15% (w/w) methacrylated gelatin (Fig. 8), it was very challenging to prepare this stock solution due to its very high viscosity. We therefore recommend using stock solutions containing 10% (w/w) methacrylated gelatin for the subsequent preparation of photo cross-linked gelatin-HydroThane™ films, as high swelling ratios were also observed for those photo cross-linked gelatin films (Fig. 8). Our finding of a decrease in $SR_{30\text{-}d\ wash}$ with increasing amounts of Irgacure, except at the highest concentration, is difficult to explain as only one sample was prepared for each experimental group. Considering our requirement for achieving the highest level of cross-linking of gelatin to prevent phase-separation, we arbitrarily selected a concentration of 10% w/w of Irgacure 651 relative to the methacrylated gelatin mass for the preparation of HydroThane™ or gelatin-HydroThane™ films.

3.2 HydroThane™ films

An experiment was conducted to determine whether the UV-irradiation procedures used to photo cross-link gelatin had any effect on HydroThane™ itself. Briefly, a 4% (w/w) HydroThane™ solution in DMSO was UV-irradiated in the Rayonet reactor at an intensity of 2 mW/cm^2 for 15 min at 365 nm, with or without the presence of 10% (w/w) Irgacure 651 relative to HydroThane™. As expected, the HydroThane™ solution remained liquid when it was irradiated in the absence of the photo-initiator in the mixture. In contrast, a clear solid film was formed following irradiation of the Irgacure 651-containing elastomer mixture, a thin layer of yellow liquid remaining at the air interface. When washed in double distilled water for one month, the HydroThane™ films shrank into a white, opaque misshapen disc. Further washing of

the HydroThane™ films in DMSO for 9 days confirmed that photo cross-linking had occurred, as suggested by the fact that the HydroThane™ films did not dissolve.

The latter finding suggests that a full IPN may be formed during the irradiation process of the pre-IPN gelatin-HydroThane™ mixtures, with both polymers being cross-linked. The formation of a full IPN following the photo cross-linking procedures instead of that of a semi-IPN as proposed for the preparation of the gelatin-HydroThane™ IPN material (1) has implications for further development of our material. Indeed, an important novel aspect of the gelatin-HydroThane™ IPN material is its mesh-like design. We envisaged using the IPN gelatin-HydroThane™ optimized formulation to form fibres that would thereafter be heat-sealed to prepare mesh-like sheets. The latter would then be gathered into a three-dimensional structure to form a tube or sphere much like the polyethylene cleansing puffs currently sold in the personal hygiene market (16). The formation of a full IPN following the photo-irradiation process might compromise the thermoplastic properties of HydroThane™. Experiments are currently underway to verify this hypothesis using differential scanning calorimetry, a method frequently used to characterize the properties of new biomaterials (17). Furthermore, we are conducting a literature review to identify alternative methods to heat-sealing for preparing the gelatin-HydroThane™ IPN fibres.

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4. Formation of photo cross-linked gelatin-HydroThane™ IPN films

This section presents a series of experiments carried out to determine the effect of altering different factors involved in the preparation of our biopolymer-elastomer IPN material on its physico-chemical properties, including its absorbency, strength, and ability to deliver a drug.

4.1 Concentration of photoinitiator

The presence of the photoinitiator in the pre-IPN mixture is essential to the successful formation of the photo cross-linked film. An experiment was thus designed to determine the effect of varying the amount of Irgacure 651 in the pre-IPN mixture on the swelling ratio of the resulting photo cross-linked gelatin-HydroThane™ films. Samples of 1 MAAH methacrylated gelatin were prepared in the absence of DMAP and TEA (see Section 2.1.1 for details), and dialysed at room temperature. All pre-IPN mixtures were prepared by mixing aliquots of 7.5% methacrylated gelatin (w/w) and 4% HydroThane™ (w/w) in scintillation vials, the ratio of these two polymers being maintained at 50:50. Irgacure 651 was then dissolved in DMSO at 5 %, 10 %, or 20% (w/w), and a 0.1-g sample of the Irgacure solution was added to the methacrylated gelatin-HydroThane™ mixtures (n=2 per experimental group). The pre-IPN gelatin-HydroThane™ mixtures were then photo cross-linked, and further processed as described in Section 3.1. Films were washed in double distilled water at room temperature and 37°C for 4 days before measuring their swelling ratio, as described in Section 3.1. Tensile strength was measured using a Zwick materials testing machine (TCFR005TN.A50, Zwick USA, Kennesaw, GA), at a test speed of 50 mm/min. The stress was calculated as the breaking force divided by the cross-section area of the film.

Figure 9 shows that increasing the concentration of Irgacure-651 from 5% to 20% reduced the swelling ratio by 9%, indicating an increased cross-linking. However, doubling the concentration of the photoinitiator reduced the strength of the film by 28% (Fig. 10). The latter finding might be explained by a greater phase-separation of the polymers at the higher concentration of photoinitiator. These findings are in agreement with reports that high concentrations of Irgacure 651 adversely affected the degree of polymerization of the biomaterials produced (18).

It is noteworthy that the curing process of the photo cross-linked films might still proceed after their removal from the reactor, due to the presence of residual free radicals. A study should therefore be conducted to assess the effect of increasing the time interval between the end of the irradiation cycle and the washing period of the photo cross-linked gelatin-HydroThane™ films. Our preliminary data indicates that the concentration of photoinitiator in the pre-IPN gelatin-HydroThane™ mixture may affect the outcome of the photo cross-linking process, albeit to a small extent (data not shown).

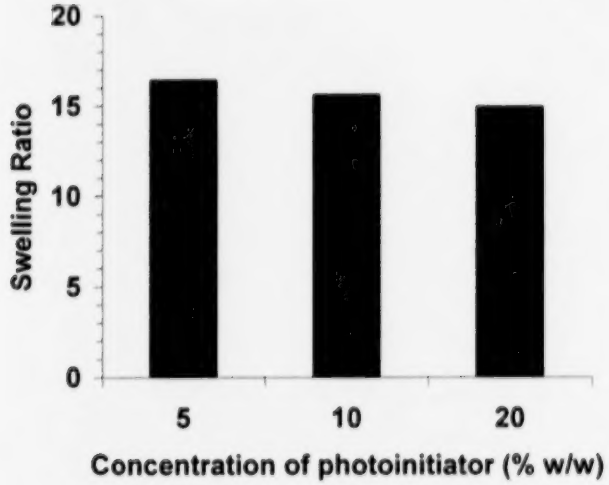

Figure 9. *Swelling ratios of gelatin-HydroThane™ films (prepared with varying amounts of photoinitiator) after a 4-d washing period in distilled water maintained at 37°C. Data are expressed as means (n=2 per group).*

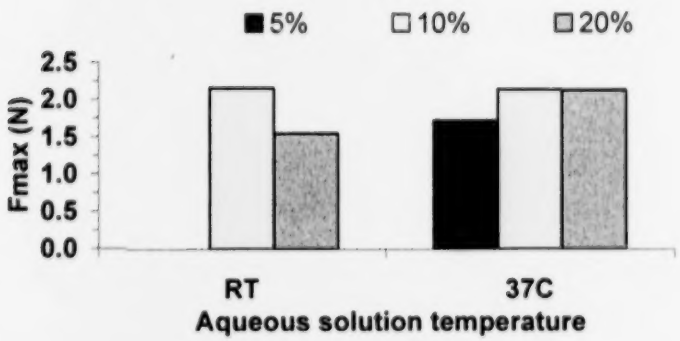

Figure 10. *Strength of gelatin-HydroThane™ films (prepared with varying amounts of photoinitiator) after a 4-d washing period in distilled water maintained at either room temperature (RT) or 37°C. Data represent n=1 per group.*

4.2 Concentrations of the stock polymer solutions and component fractions of gelatin and HydroThane™

Gelatin and HydroThane™ have two distinct roles in our IPN material, namely, providing absorbency and strength, respectively. Studies were therefore carried out to determine to what extent altering the amount and proportions of these polymers in the pre-IPN mixture would affect these properties. Samples of 1 MAAH methacrylated gelatin were prepared in the absence of DMAP and TEA (see Section 2.1.1 for details), and dialysed at room temperature. Pre-IPN mixtures were prepared in DMSO by mixing in a scintillation vial varying amounts of stock solutions of methacrylated gelatin (5%, 7.5%, and 10% w/w) and HydroThane™ (2%, 3%, 4%, and 5% w/w). The ratio of HydroThane™ to gelatin was arbitrarily maintained at 1:1 for all photo cross-linked IPN films. An aliquot of 10% w/w Irgacure 651 was then prepared in DMSO, and added to the gelatin-HydroThane™ pre-IPN mixtures. The different mixtures were then vigorously vortexed for 30 s; purged with nitrogen for 5 min; and, UV-irradiated (350 nm) at an intensity of 2 mW/cm^2 for 15 min. The resulting gelatin-HydroThane™ IPN films were cooled at room temperature for 60 min, before being washed in double distilled water at room temperature for up to 20 days. Frequent water changes were performed during the washing period. The swelling ratio of the IPN films was calculated as previously described.

No photo cross-linking occurred when using 2% (w/w) HydroThane™, either after increasing the irradiation time to 60 min or increasing the amount of photoinitiator (data not shown). Irradiation of the pre-IPN mixtures containing 3% (w/w) HydroThane™ for 15 min yielded semi-solid films, even when further increasing the amount of photoinitiator in these mixtures. However, solid IPN films were produced when increasing the duration of the irradiation to 60 min. All pre-IPN mixtures containing at least 4% HydroThane™ were successfully cross-linked after 15 min of irradiation. This data might indicate that the viscosity (and concentration) of the HydroThane™ solution plays a role in the formation of photo cross-linked gelatin-HydroThane™ IPN films.

Figure 11 depicts the changes in swelling ratios of the photo cross-linked gelatin-HydroThane™ films prepared with different proportions of the biopolymer and elastomer. Many of the gelatin-HydroThane™ IPN films prepared had swelling ratios above our target value of 15, the latter figure arbitrarily chosen to exceed by 50% the values reported for most commercially available wound dressings. It is noteworthy that all films had hydration values above 0.9 (data not shown), a highly desirable characteristic for our application of this IPN material as a wound dressing (15). However, it is unclear from the current series of experiments whether or not the subsequent step of freeze-drying the gelatin-HydroThane™ IPN material after completion of the washing procedures, in preparation for its packaging, will affect its absorbency. The swelling ratios of all IPN films increased gradually during the first 8 days of the washing period. Moreover, photo cross-linked gelatin-HydroThane™ IPN films prepared with initial concentrations of 3% (w/w) HydroThane™ and 7.5% (w/w) gelatin tended to yield higher swelling ratio values after 8 days of washing than any other IPN films (Fig. 11). However, the former IPN films appeared considerably

weaker. Our findings suggest that parameters other than the swelling ratio (e.g., mechanical strength) should be taken into account for selecting the optimal composition of the biopolymer-elastomer material. It is noteworthy that there was no relationship between the total amount of polymer in the pre-IPN solution and either the hydration or swelling ratio of the photo cross-linked IPN films.

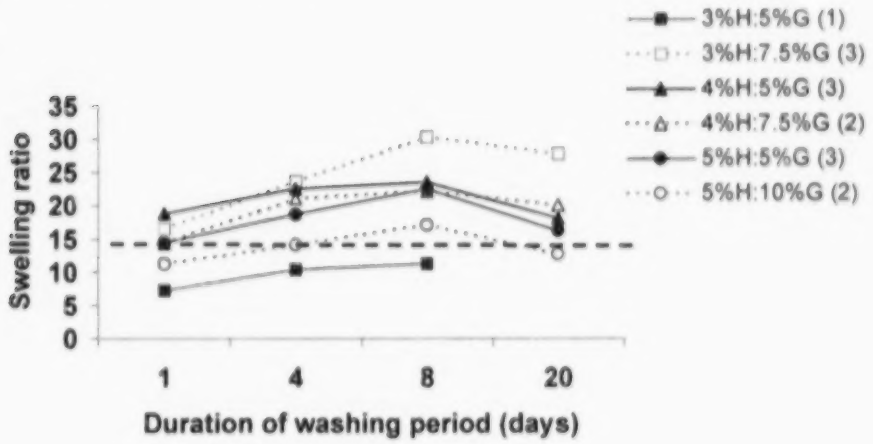

Figure 11. *Swelling ratios of photo cross-linked gelatin-HydroThane™ films prepared with different proportions of gelatin (G) and HydroThane™ (H) at different time intervals following immersion in double distilled water. Dashed line represents the swelling ratio targeted for our application of the IPN material as a wound dressing. Data are expressed as means (sample size in parenthesis). SEM are not shown for the sake of clarity.*

A gradual reduction in swelling was observed for all IPN films during the last 12 days of the washing period (Fig. 11). To provide a better understanding of this long-term reduction in swelling, three of the IPN films that showed a relatively constant swelling over the 20-d period were dried at 50°C. Assuming that HydroThane™ did not significantly degrade during the immersion, we estimated that only 50% of the initial amount of methacrylated gelatin used to prepare these IPN films was recovered. The maintenance of the hydration status of these films despite a reduction in their gelatin content suggests that any swelling observed in the initial period of washing of the IPN films may be limited by the physical space available for the gelatin to expand within the gelatin-HydroThane™ network.

The observed loss of the gelatin may represent the long-term dissolution and degradation of gelatin in the IPN films, processes that could be confirmed by analysing the composition of the washing water at different time intervals using gel permeation chromatography. Whether this degradation is due to the action of bacteria or hydrolysis *per se* is unknown. Though random aliquots of the washing media did not show any bacterial contamination (data not shown), addition of 1% sodium azide to the washing solution as a preservative agent is recommended during the washing

period. Our findings also suggest that the washing period of the gelatin-HydroThane™ IPN films should be less than 8 days, to minimize the loss of gelatin. Alternately, studies are currently underway to determine the stability of the gelatin-HydroThane™ IPN films in methanol.

It is likely that the gelatin loss from our gelatin-HydroThane™ IPN material will be exacerbated upon its exposure to a biological fluid compared to that observed following immersion in double distilled water due to the various hydrolytic enzymes present in body fluids. Furthermore, while the washing procedure was performed at room temperature, there is no indication how absorption of warmer wound exudates (i.e., approximately 33°C) will affect the gelatin fraction in the material. Studies are currently carried out to assess the extent of gelatin loss when the biopolymer-elastomer material is hydrating for various time intervals in a 50% fetal calf serum solution maintained at 37°C.

All gelatin-HydroThane™ IPN films prepared in this section contained equal fractions of the two polymers. While the HydroThane™ component is critical to maintain the elasticity and strength of the material, its concentration should not be increased so much as to reducing either the hydration values of our IPN films below 0.8 (to meet the absorption requirements of these films as a wound dressing material; 15) or the swelling ratios below our target value of 15. A preliminary experiment was thus conducted in which IPN films containing component fractions of the two polymers of 30G:70H, 50G:50H and 70G:30H (gelatin:HydroThane™; n=1 per experimental group) were prepared. Each film was prepared using 7.5% (w/w) methacrylated gelatin (prepared as described previously in Section 4.1) and 4% (w/w) HydroThane™, and were photo cross-linked as described above.

Not surprisingly, the 70G:30H IPN film was weaker and more fragile than the other films. It swelled to about twice its diameter after a few days of washing in double distilled water, closely resembling a photo cross-linked gelatin film. However, it also rapidly degraded during the washing procedures. In contrast, the 30G:70H IPN film had visually smaller domains than the 50G:50H film, and appeared stronger. While both the 30G:70H and the 50G:50H films yielded hydration values of 0.94 (data not shown), the swelling ratio was lower for the former film (15 vs. 20). Based on our findings, it appears that the combination of 5-7.5% (w/w) methacrylated gelatin and 4-5% (w/w) HydroThane™ at a ratio of 50:50 constitutes the optimum conditions for preparing an IPN material with the target swelling ratio.

Interestingly, phase separation of the pre-IPN mixtures during domain formation was clearly apparent in all three IPN films, regardless of the component fractions of the two polymers. It is noteworthy that more HydroThane™ seemed to be present at the air interface of the IPN films, while gelatin formed a film along the glass interface, suggesting that the polymers may not have been cross-linking quickly enough to prevent this phase separation. Different procedures for mixing the two polymers were therefore assessed.

4.3 Mixing conditions of the pre-IPN mixture

The gelatin and HydroThane™ components in the pre-IPN solution must be thoroughly mixed prior to initiating the photo cross-linking process to minimize phase separation. While mixing the pre-IPN solution while the UV irradiation is proceeding would likely minimize phase-separation, such procedure proved technically impossible to achieve. In fact, UV-irradiation typically proceeded within 30 min of vortexing the two polymer solutions; the resulting IPN films are referred to as 'VORTEX'. A preliminary experiment was carried out to assess the effect of two additional mixing methods. Firstly, samples of 1 MAAH high molecular weight Type A gelatin (Bloom number of 300; Great Lakes Gelatin Inc., Grayslake, IL) were methacrylated in the absence of DMAP and TEA, and dialysed at approximately 40°C (see Section 2.1.1 for details). Pre-IPN mixtures were then prepared by mixing in a Teflon-lined scintillation vial 7.5% methacrylated gelatin (w/w) and 4% HydroThane™ (w/w), the ratio of these two polymers being maintained at 50:50. An aliquot of Irgacure 651 (10% w/w) was then added to the methacrylated gelatin-HydroThane™ pre-IPN mixtures. All mixtures were gently mixed on a rocker at room temperature for 3 or 6 days before photo cross-linking proceeded as described in Section 3.1. These IPN films are referred to as 'PROLONGED MIXING' (n=1 per time interval). Secondly, one IPN film was prepared by: adding methacrylated gelatin in a 4% (w/w) HydroThane™ solution to a final concentration of 7.5% (w/w) methacrylated gelatin; stirring the mixture until a homogenous solution was obtained; and, photo cross-linking the pre-IPN mixture. This IPN film is referred to as 'GELATIN INTO HYDROTHANE'. All IPN films were then washed in distilled water for 4 days to remove all non-reacted reagent. The gelatin-HydroThane™ IPN material will likely be commercially available in a semi-hydrated form, to allow for maximal absorption of wound exudates as well as to maximize the compressibility of the material. The washed films were therefore freeze-dried at room temperature for 5 days; and, immersed for 2 days in serum maintained at 37°C to simulate a condition where the wound dressing would remain on the wound undisturbed. The re-hydrated films were then cut into strips (10 mm wide x 20 mm long x 2 mm thick), and their tensile strength measured as described in Section 4.1. The stress was calculated as the breaking force divided by the cross-section area of the film.

The gelatin-HydroThane™ film photo cross-linked after a 6-d mixing period appeared visually more homogeneous than the one formed after only 3 days, the latter also showing bigger domains. One might hypothesize that a longer mixing time allows for a greater interaction and diffusion of the polymers in solution, therefore leading to an increased hydrogen bonding and better cross-linking. While clear and homogeneous pre-IPN solutions were observed when prolonging the period of mixing to 6 days, the pre-IPN GELATIN INTO HYDROTHANE mixture remained turbid for the entire mixing period. However, the GELATIN INTO HYDROTHANE IPN film had smaller domains than the 6-d PROLONGED MIXING IPN film. Despite these apparent morphological differences, there were no significant differences in the absorbency of the films prepared using the different methods (data not shown). Moreover, the improvement in homogeneity of the pre-IPN PROLONGED MIXING and GELATIN INTO HYDROTHANE mixtures did not translate into a corresponding increase in strength of these IPN films (Fig. 12).

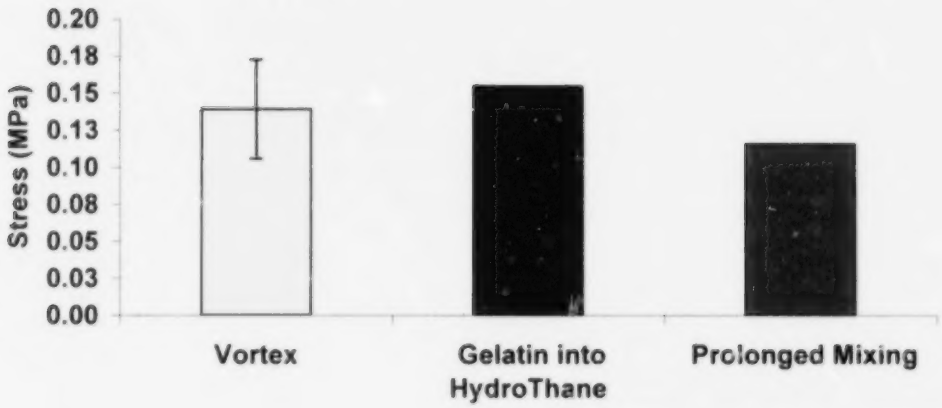

Figure 12. *Breaking stress of photo cross-linked gelatin-HydroThane™ IPN films re-hydrated for 2 days in serum maintained at 37°C. Three different methods were used for mixing the polymers in the pre-IPN mixture. Data represent n=1 per experimental group except for Vortex (means ± SEM; n=3).*

An attempt was made to assess whether these different mixing methods had any impact on another parameter that is essential for using our gelatin-HydroThane™ IPN material as a wound dressing, that is, the ability of the biomaterial to deliver an antiseptic agent. IPN films were prepared as described above (n=3 per mixing method); re-hydrated for 48 h in a 5% aqueous solution of mafenide acetate (Sigma Chemical, St-Louis, MO); and, gently patted-dry. Mafenide acetate-loaded Allevyn™ dressings (Smith & Nephew, Lachine, QC) were prepared by immersing each sample for 30 min in the aqueous drug solution. The drug-loaded Allevyn™ dressings were then squeezed under a sterile custom-built bench-top rolling press for 60 s, to retain a standardized amount of the drug solution. All samples (i.e., IPN films and Allevyn™) were then cut into 20-mm^2 discs (n=3 per experimental group).

The *in vitro* bactericidal activity of these samples was then assessed using a standard Kirby-Bauer assay. Briefly, *Ps. aeruginosa*-coated beads (American Type Culture Collection no. 27317) were placed in 20 mL of sterile TSB (VWR, Mississauga, ON). The bacterial strains were grown at 37°C in nutrient broth for 18 h in a shaking water bath to obtain a log-phase growth culture. Cultures were then centrifuged (2500 rpm; 20 min; 4°C), and the supernatant washed three times with PBS. The inoculum was then re-suspended in sterile PBS to a final dilution of approximately 10^9 CFU per mL. Serial dilutions were plated on Trypticase Soy agar enriched with 5% sheep blood to assess bacterial concentrations in the inoculum. A 100-uL aliquot of a 10^6 Colony Forming Units bacterial inoculum was then streaked onto a Mueller-Hinton agar plate, and the plates were allowed to air-dry for 10 min. Individual mafenide acetate-loaded dressings were centered on the inoculated agar. A sterile metal O-ring was then placed on top of each dressing to ensure its contact with the agar, and the plates were incubated overnight at 37°C. Corrected zones of inhibition were measured as the

difference between the growth area around the edges of the dressings and the corresponding surface area of the dressing. After measurement, the dressings were transferred aseptically to freshly inoculated Mueller-Hinton agar, and the procedures repeated until no zone of inhibition was observed.

Figure 13 depicts the *in vitro* bactericidal activities of the IPN films prepared using various mixing methods compared to that of Allevyn™. VORTEX and GELATIN INTO HYDROTHANE IPN films showed similar bactericidal activities during the first three days, the latter films being bactericidal for up to 4 days. While the *in vitro* bactericidal activity of the PROLONGED MIXING films was approximately 10% greater than that of the other types of films for the first two days, this difference had disappeared after 3 days. Moreover, the *in vitro* bactericidal activity of antiseptic-loaded photo cross-linked IPN films was far superior to that of the commercial polyurethane-based wound dressing, regardless of the mixing method selected for mixing the polymers in the pre-IPN mixture. Considering our findings of comparable drug deliveries of the different IPN films over the period of intended application of the material as a wound dressing (i.e., 3 days), we recommend to vortex the pre-IPN mixture for the subsequent preparation of the biomaterial to simplify this procedural step.

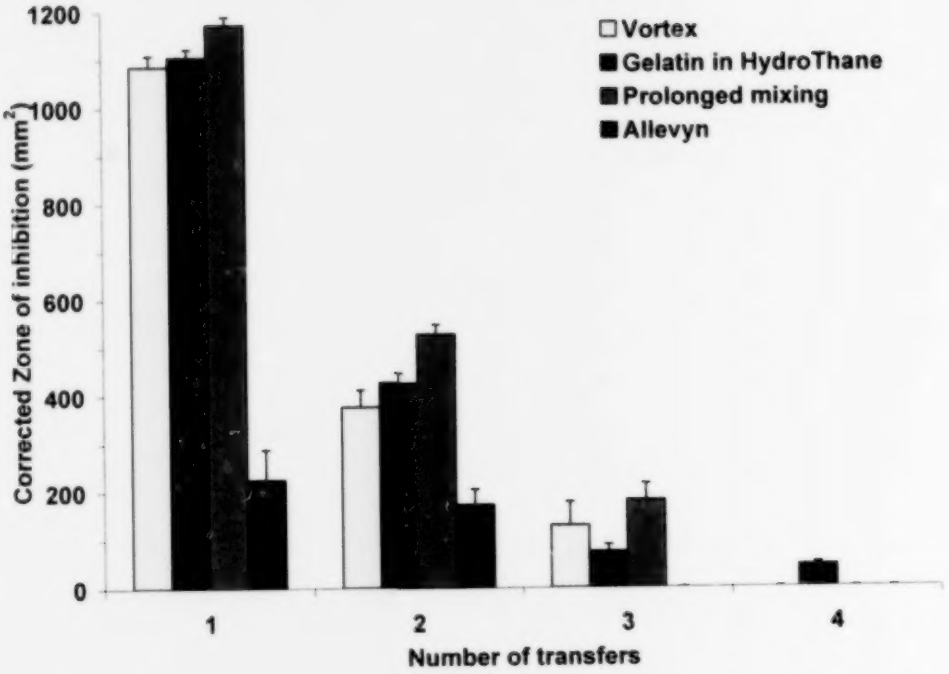

Figure 13. *In vitro bactericidal activity of mafenide acetate-loaded Allevyn™ and photo cross-linked gelatin-HydroThane™ IPN films prepared using various methods for mixing the polymers. Data represent means ± SEM (n=3).*

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5. Scale-up of photo cross-linked film production

Although the photo cross-linking procedures described insofar were successful for preparing gelatin, HydroThane™, and gelatin-HydroThane™ IPN films, the process was relatively inefficient as only one film could be photo cross-linked every 25 min. An attempt was made to increase the rate of film production by stacking scintillation vials within the UV irradiator. However, we noticed that the polymer solution in the top vial was consistently less cured than that in the bottom vial. Another technical difficulty was that some of the films photo cross-linked in the Rayonet reactor could not be used for the planned experiments, as they tended to adhere to the bottom of the scintillation vials, and were therefore damaged upon their retrieval. A new irradiation system was therefore purchased in an attempt to overcome these technical difficulties. This new system (AB-Manufacturing Inc., Model AB-M Series 60 Exposure Systems, San Jose, CA) uses a top-down irradiation pattern instead of a cylindrical one such as that in the Rayonet reactor. The light appears highly uniform in intensity and is collimated with an angle divergence of a maximum of 2 degrees. While we have measured an average irradiation intensity of 36.6±0.5 mW/cm^2 (n=16 spots across the irradiated area), the addition of a plastic film to protect the working polymer solution reduces this value to 33 mW/cm^2. Up to 16 films could be prepared in 25 min using the AB-M reactor.

A 4% (w/w) HydroThane™ solution in DMSO was UV-irradiated at an intensity of 33 mW/cm^2 for 15 min at 365 nm in the presence of 10% (w/w) Irgacure 651. Although we hypothesized that the higher intensity output in the new irradiator (i.e., approximately 16 times that of the Rayonet reactor) would increase the rate of the photo cross-linking reaction, the HydroThane™ films did not cross-link. However, continuous purging of the HydroThane™ solution with nitrogen during the UV-irradiation procedures produced cross-linked elastomer films, no residual solution being present at the surface of the film as previously observed using the Rayonet reactor. These findings suggest that HydroThane™ is sensitive to the presence of oxygen during the UV-irradiation process. Nevertheless, the fact that HydroThane™ cross-linked using the Rayonet reactor without simultaneous nitrogen purging also suggested that other factors, likely related to the open-design of the new reactor as well as its higher intensity output, might determine the outcome of the UV irradiation process.

5.1.1 Temperature of reactor

Since the AB-M reactor appeared to be better ventilated than the Rayonet, we hypothesized that the difference in photo cross-linking between the two apparatus might be temperature-related. Tests were carried out to provide an understanding of the effect of temperature on the photo cross-linking process. Briefly, a thermistor was placed in each of the UV exposure systems (in close proximity to the pre-IPN mixture) to measure the ambient air temperature during the 15-min UV-irradiation period. Two-mL aliquots of distilled water

(at room temperature) or of gelatin-HydroThane™ IPN mixtures were then placed in the Teflon mould to examine any differences in the heat transfer process to the aqueous solution.

Figure 14 shows that the ambient temperature in the AB-M irradiator sharply increased to 37°C within 60 s, then gradually increasing to 40°C for the remainder of the 15-min irradiation period. In contrast, the air temperature in the Rayonet never increased above 34°C. While comparable increases in the water temperature were observed regardless of the irradiator used (Fig. 14), the temperature of the pre-IPN mixture remained approximately 3.5 °C higher throughout the experiment for the samples irradiated with the AB-M reactor compared to those in the Rayonet (Fig. 15).

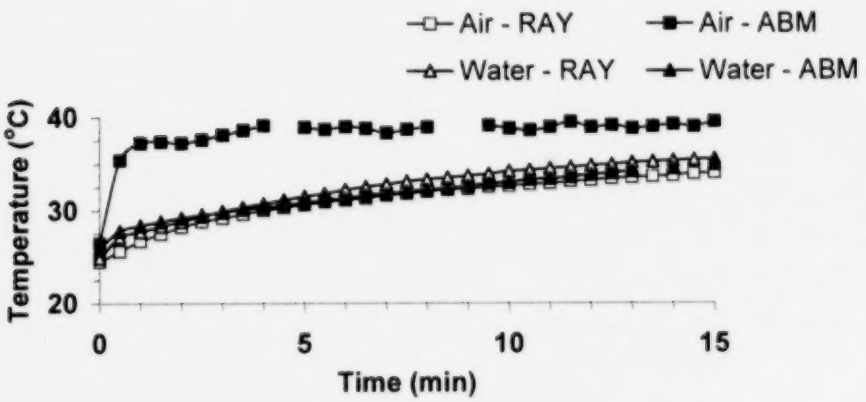

Figure 14. *Changes in ambient temperature in the chamber of the Rayonet (RAY) and AB-M reactors (ABM) during a 15-min irradiation period of a 2-mL sample of water. Data represent n=1 per experimental condition.*

We then designed an experiment to assess whether warming up the gelatin-HydroThane™ IPN mixture and/or mould used to cast the films for 30 min at 50°C prior to completion of the UV-irradiation procedures in the AB-M reactor would affect the strength of the photo cross-linked films. Pre-IPN mixtures (n=3 per experimental group) were also irradiated for 15-min at 365 nm using the Rayonet. The resulting gelatin-HydroThane™ films were cut into strips and their tensile strength was measured after a 4-d immersion in 1% sodium azide aqueous solution (for details, see Section 4.1). Figure 16 shows that the Rayonet-produced films were three times stronger than those photo cross-linked using the AB-M reactor. Pre-heating the Teflon mould partially restored the strength of the AB-M films. However, pre-heating both the mould and the pre-IPN mixture further increased their strength, the AB-M cross-linked films being 35% stronger than those irradiated in the Rayonet reactor (Fig. 16). Thus, our findings suggest that the temperature *per se* contributes significantly to the efficacy of the photo cross-linking procedures

in the AB-M irradiator, likely due to the fact that the rate of production of radicals from Irgacure is highly temperature-dependent.

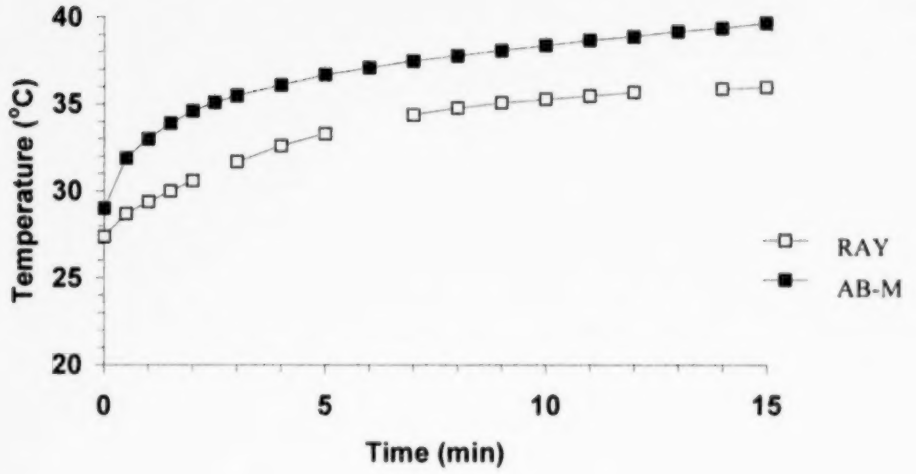

Figure 15. *Changes in temperature of a gelatin-HydroThane™ IPN mixture irradiated for 15-min using either the Rayonet (RAY) or AB-M reactor. Data represent n=1 per experimental condition.*

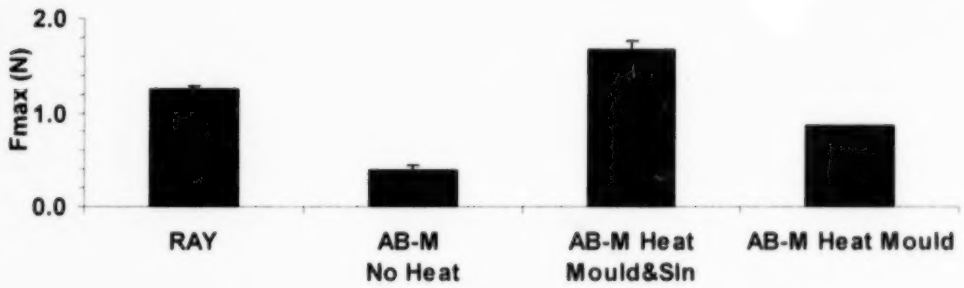

Figure 16. *Strength of gelatin-HydroThane™ films photo cross-linked in the Rayonet (RAY) or AB-M reactor. Strength was measured after a 4-d immersion in 1% sodium azide aqueous solution. The mould and/or pre-IPN mixtures were pre-warmed for 30 min at 50°C prior to the irradiation procedures. Data represent means ± SD, n=3 per experimental condition, except for ABM Heat Mould (n=2).*

5.1.2 Intensity output

Several aliquots of Irgacure 651 were dissolved in DMSO to a final concentration of 10% w/w, and irradiated at 365 nm for 15 min using either the Rayonet or the AB-M reactor. Since the intensity output of the AB-M could not be manually adjusted to the same level as that observed for the Rayonet (i.e., 2 mW/cm^2), layers of Plexiglas® sheets (1.5 mm thick) were used to cover the UV source. The relationship between the intensity output measured by the photometer (AB-Manufacturing Inc., Model 100, San Jose, CA) and the number of sheets used is shown in Figure 17. The decomposition of the photoinitiator was then examined using UV-visible spectrophotometry. Figure 18 shows that the Irgacure 651 was decomposed at a much faster rate in the AB-M reactor with the original UV light configuration than in the Rayonet reactor, likely due to the 16-fold greater irradiation output of the former. However, reducing the irradiation intensity to approximately 6 mW/cm^2 produced a similar spectrum to that observed for the samples irradiated at 2 mW/cm^2 with the Rayonet. The latter finding might be related to an underestimation of the irradiation intensity in the Rayonet, due to a difference in geometry between the reactor and the UV probe (i.e., cylindrical vs. flat). Alternately, inherent differences in the output spectra produced by the two systems (i.e., entire UV spectrum for the AB-M vs. single output spectrum for the Rayonet) might also have played a role. Interestingly, intensity outputs below 2 mW/cm^2 yielded decomposition patterns comparable to those of the non-irradiated photoinitiator.

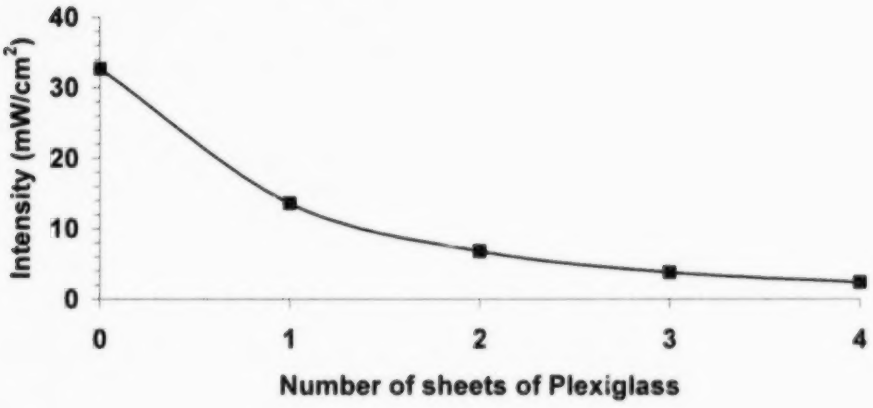

Figure 17. *Relationship between the intensity output of the AB-M reactor and the number of sheets of Plexiglas® covering the UV light.*

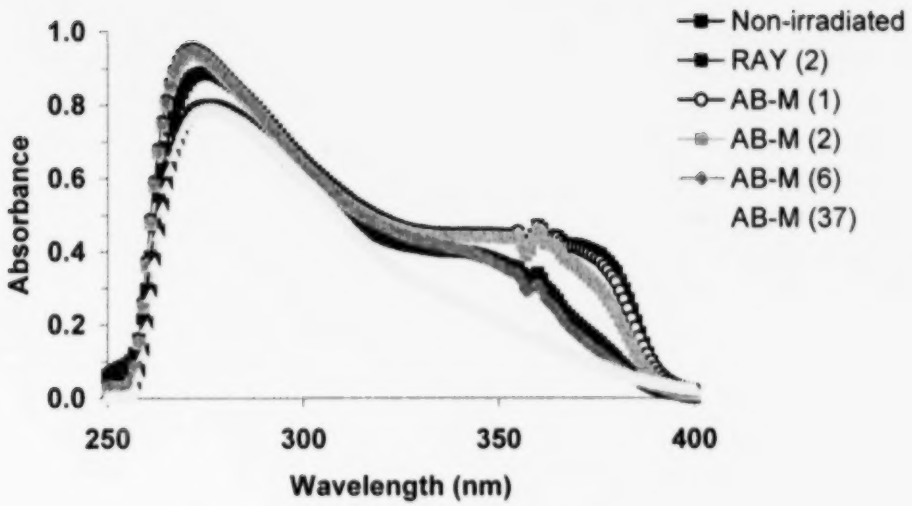

Figure 18. *UV spectra of Irgacure 651 before and after irradiation for 15 min at 365 nm using either a Rayonet (RAY) or an AB-M reactor. Number in brackets indicates the approximate intensity of irradiation (expressed in mW/cm^2).*

The effect of the irradiation output on the strength and absorbency of freeze-dried gelatin, HydroThane™, and gelatin-HydroThane™ IPN films was then assessed. Briefly, several aliquots of solutions containing either 4% (w/w) HydroThane™ or 7.5% (w/w) gelatin methacrylated in DMSO were prepared. A mixture of the two polymers was also prepared at a ratio of 1:1, and is referred to as the pre-IPN mixture. Aliquots of the polymer stock solutions as well as of the pre-IPN mixture were then UV-irradiated at different intensity outputs (n=1 per experimental group). All films were then washed in distilled water for 4 days; freeze-dried at room temperature for 5 days; and, immersed for 7 days in serum maintained at 37°C. The tensile strength of some of the resulting films was measured as described previously in Section 4.1. The stress was calculated as the breaking force divided by the cross-section area of the film. The strain of the films, that is, a measure of their elasticity, was calculated as the ratio of the elongation of the strip over its original length. More films were washed in distilled water for 3 days, the swelling ratio being calculated as the ratio of the wet weight after the 3-d washing period over the initial dry weight.

There was no effect of altering the intensity output of the AB-M reactor on the stress of both the photo cross-linked gelatin films and gelatin-HydroThane™ IPN films (Fig. 19). In contrast, the stress of the HydroThane™ films increased as a function of the intensity output, reaching peak values between 4-7 mW/cm^2. Similarly, the elasticity of all types of films peaked at these intensity outputs (Fig. 20).

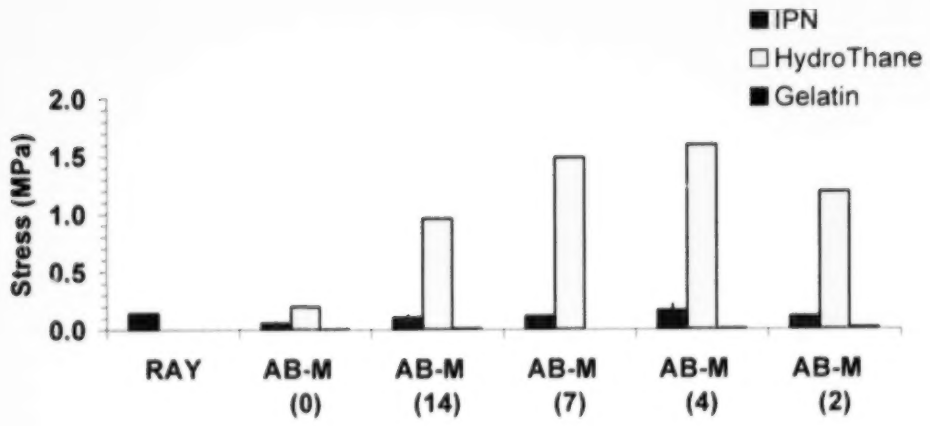

Figure 19. *Effect of the intensity output of the AB-M reactor on the stress of freeze-dried gelatin, HydroThane™, and gelatin-HydroThane™ IPN films (IPN) re-hydrated for 7 days in serum maintained at 37°C. Only IPN films were photo cross-linked using the Rayonet reactor (RAY). The intensity output is indicated in parenthesis (mW/cm²). Data represent n=1 per experimental condition except for IPN films (means ± SEM, n=3).*

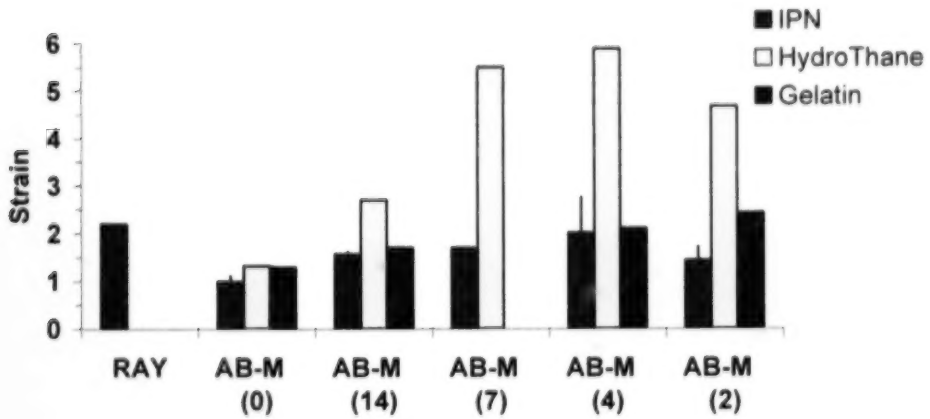

Figure 20. *Effect of the intensity output of the AB-M reactor on the elasticity of freeze-dried gelatin, HydroThane™, and gelatin-HydroThane™ IPN films (IPN) re-hydrated for 7 days in serum maintained at 37°C. Only IPN films were photo cross-linked using the Rayonet reactor (RAY). The intensity output is indicated in parenthesis (mW/cm²). Data represent n=1 per experimental condition except for IPN films (means ± SEM, n=3).*

There was no effect of altering the irradiation intensity on the swelling ratios of the HydroThane™ films and gelatin-HydroThane™ IPN films photo cross-linked at the different output intensities (Fig. 21). However, our findings of swelling ratios of up to 2.5 for the photo cross-linked HydroThane™ films are in contrast to the manufacturer's claim that HydroThane™ can only absorb up to 25% of its dry weight. This discrepancy might related to the fact that cross-linked HydroThane™ forms porous structures that trap water. Nevertheless, these findings suggest that the elastomer component might also contribute to the overall absorbency of our IPN material.

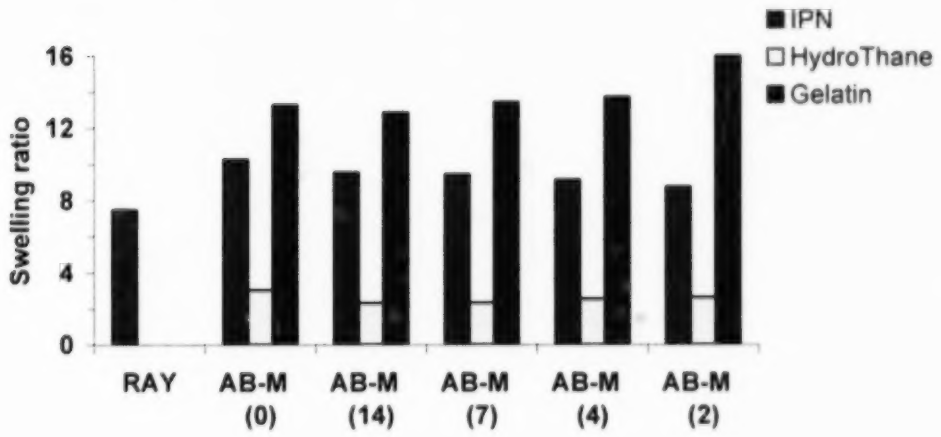

Figure 21. *Effect of the intensity output of the AB-M reactor on the swelling ratio of freeze-dried gelatin, HydroThane™, and gelatin-HydroThane™ IPN films (IPN) re-hydrated for 3 days in serum maintained at 37°C. IPN films were also photo cross-linked using the Rayonet reactor (RAY). The intensity output is indicated in parenthesis (mW/cm²). Data represent n=1 per experimental condition.*

A higher intensity output should have intuitively led to a greater cross-linking of the elastomer. Our findings of the reverse trend might be explained by considering the mechanism of action of Irgacure 651. Indeed, UV irradiation of Irgacure 651 generates two primary free radicals, the benzoyl radical initiating the polymerization process while the dimethoxy benzyl radical terminates it (18; Fig. 2). It is possible that the higher irradiation intensity output of the AB-M irradiator produced a greater amount of dimethoxy benzyl, thus prompting the end of the reaction and a reduced cross-linking of HydroThane™. Alternately, the decomposition of the Irgacure 651 into the different free radicals might also have been much faster than their consumption through polymerization of HydroThane™, thereby leading to the reaction of free radicals with each other to form UV-stable molecules. As a result, less photoinitiator might have been available for polymerization of HydroThane™ and thus

successful formation of a film. Based on our experimental data, we recommend irradiating Hydrothane™-based reaction mixtures at intensities ranging between 4-7 mW/cm^2 when using the AB-M reactor for scaling up the production of IPN material for *in vitro* or *in vivo* assessment of its properties.

6. Conclusions

The present technical report describes the development of a photo-cross-linking method for preparing the gelatin-HydroThane™ material. Several factors playing a role in the hydration and mechanical properties of our biomaterial were identified, allowing the optimization of the IPN formulation for preparation of films. The production of films was also scaled-up to enable the comprehensive *in vitro* and *in vivo* evaluation of the medicated biomaterial.

While IPN films with desirable swelling ratios were produced, a number of challenges remain to be addressed. The reaction apparatus is currently a scintillation vial with a rubber septum or a Teflon® mould covered with Plexiglas® sheets. Thus, one surface of the IPN film is in contact with either glass or Teflon® while the other is in contact with air. The effect of these two interfaces on the final properties of the IPN will be apparent when we examine the morphology of the IPN. If one of the surfaces has very minimal distribution of gelatin domains, it will directly affect the performance of the film with regard to its hydration and drug release properties. Modifications to the reaction apparatus should then be made so that the interface promotes a uniform distribution of gelatin domains throughout the IPN film.

The freeze-drying procedures leading to the preparation of the dry gelatin-HydroThane™ IPN material reduced significantly its ability to absorb serum. While the swelling ratios of re-hydrated IPN films were comparable to those of commercial hydrophilic polyurethane dressings, they corresponded to only 66% of our original target. Different strategies should be therefore investigated to improve the maximum absorption of our prototype biomaterial, including alterations in the freeze-drying conditions, increases in the component fraction of gelatin in the pre-IPN mixture, or incorporation of highly absorbent monomers such as acrylamide.

One of the novelties of the biopolymer-elastomer biomaterial proposed under the TIF is the configuration of the IPN material into a tri-dimensional open mesh design. Indeed, we believed that the mesh design would be more compressible than conventional dressing materials, thus minimizing the size of the dressing and storage requirements. While we successfully prepared gelatin-HydroThane™ IPN films, the current IPN formulations might need to be further optimized to prepare hydrophilic, elastic, fibres with appropriate structural integrity as well as drug delivery and hydration properties.

In summary, synthesis of biopolymer-elastomer composites via a photo cross-linking process was successfully achieved. Further development of the gelatin-HydroThane™ IPN biomaterial as a medicated wound dressing for treating deep, hemorrhagic cavity injuries sustained on the battlefield appears promising.

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List of symbols/abbreviations/acronyms/initialisms

D	Daltons
DMAc	Dimethylacetamide
DMAP	Dimethylaminopyridine
DMF	N,N-dimethylformamide
DMSO	Dimethylsulphoxide
DND	Department of National Defence
EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide
FTIR	Fourier Transformed Infrared Spectroscopy
GTA	Glutaraldehyde
H_2SO_4	Sulfuric acid
$H_{4\text{-d wash}}$	Weight fraction of water in the GTA cross-linked gelatin film following the 4-d washing procedures
IPN	Interpenetrating Polymer Network
MAAH	Methacrylic anhydride
mL	Milliliter
MOM	Military Operational Medicine
MPa	MegaPascal
MWCO	Molecular weight above which a certain percentage (e.g., 90 percent) of the solute in the dialysis solution is retained by the membrane
N	Newton
NMR	Nuclear magnetic resonance

PBS	Phosphate Buffered Saline
rpm	Revolutions per minute
SD	Standard deviation of the mean
SEM	Standard error of the mean
$SR_{4\text{-d wash}}$	Swelling ratio of the GTA cross-linked gelatin film following the 4-d washing procedures
TEA	Triethylamine
UV	Ultraviolet
WBE	Work Breakdown Element
w/w %	Weight of solute per weight of solvent, expressed as a percentage

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(U) This report describes the process for photo cross-linking the components of a biopolymer-elastomer interpenetrating polymer network (IPN) biomaterial for use as a wound dressing. Cross-linking of methacrylated gelatin was performed by ultraviolet irradiation in the presence of a photoinitiator. The yield and extent of the gelatin methacrylation reaction were quantified using various methods. Unexpectedly, we determined that HydroThaneTM also cross-linked during the polymerization process, suggesting that a full IPN may be formed during the ultraviolet irradiation of the pre-IPN gelatin-HydroThaneTM mixtures. Photo cross-linking of pre-IPN mixtures containing methacrylated gelatin and HydroThaneTM produced films with desirable swelling ratios for our intended application. Several factors were examined for their contribution in determining the absorbency and/or mechanical strength of the films. These factors include: concentration of the photoinitiator; concentrations and component fractions of the different polymers; and, mixing conditions of the pre-IPN mixture. We also determined that antiseptic-loaded photo cross-linked IPN films remained bactericidal for up to 3 days. Lastly, the production of IPN films was scaled up to enable future in vitro and in vivo testing of IPN films. In summary, further development of the gelatin-HydroThaneTM IPN biomaterial as a medicated wound dressing for treating deep, hemorrhagic cavity injuries sustained on the battlefield appears promising.

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(U) interpenetrating polymer network; biopolymer; elastomer; hydration